

CONNECTIVE TISSUE ORIGIN OF THE AMYLOID-RELATED PROTEIN SAA*

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A unique protein component, protein AA,¹ has been isolated from amyloid fibrils deposited in secondary amyloidosis (1-4), and also in some cases of primary and myeloma- or macroglobulinemia-associated amyloidosis (5). Antisera to protein AA have been used to detect a related serum protein, SAA, which has been suggested to be a circulating precursor of the fibril protein (5-8). The observation that AA and SAA have identical N-terminal amino acid sequences (9), whereas SAA has a mol wt (approximately 14,000 daltons) about 50% greater than AA (approximately 9,000 daltons) (9-11) is consistent with this suggestion.

Protein SAA which is present in low concentration in normal sera, is increased in concentration in sera from patients with amyloidosis and also in sera from patients with diseases known to predispose to amyloidosis (5-8). Very high levels of SAA have been reported during acute inflammatory episodes (8) and after the injection of endotoxin (12), a procedure known to induce experimental amyloidosis (13-15). These observations suggest that SAA may be an acute-phase reactant or, alternatively, a normal tissue constituent released into the circulation as a result of injury associated with the inflammatory response. In the present study, evidence supporting the latter possibility has been obtained by employing indirect immunofluorescence to identify SAA-like material in fibroblasts grown in culture and in cryostat sections of different connective tissues.

Materials and Methods

Amyloid Fibril Proteins and Amyloid-Related Serum Protein SAA. Alkali-degraded amyloid fibrils (DAM)² were prepared by treatment with 0.1 N NaOH and amyloid protein AA was isolated by gel filtration of guanidine-degraded amyloid fibrils as described in detail elsewhere (2, 5).

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¹ For nomenclature see: Wegelius, O. and A. Pasternack, editors. *In Amyloidosis*. Academic Press, Inc., New York and London. In press.

² *Abbreviations used in this paper.* DAM, alkali-degraded amyloid fibrils; HEF, human embryonal fibroblasts; IFL, indirect immunofluorescence; SF antigen, fibroblast surface antigen; TAB, typhoid-paratyphoid A and B.

Amyloid-related serum protein SAA was isolated from strongly SAA-positive sera by gel filtration in 10% formic acid (9).

Anti-SAA Antibodies. Purified protein SAA was used for immunization of rabbits by intramuscular and subcutaneous injections in complete Freund's adjuvant. The resulting antisera precipitated SAA but not AA in double-diffusion tests. However, isolated protein AA or DAM completely blocked the antiserum reaction with SAA in absorption tests. Purified anti-SAA antibodies were obtained by immunoabsorption of anti-SAA antiserum using glutaraldehyde in solubilized SAA-positive sera (16).

SAA-Positive Human Sera. Sera from patients with chronic inflammatory conditions and from patients with amyloidosis were assayed for the presence of SAA by double diffusion against anti-SAA antiserum and quantitated by radial diffusion as previously described (6). These SAA-positive and negative sera were used in immunofluorescence inhibition assays. Serum from one of us (R. A.) was also tested before and after typhoid-paratyphoid A and B (TAB) vaccination, a procedure which strongly stimulated SAA production (12).

Human Tissues. The fetal tissues used as substrates for the immunofluorescence assays were obtained from medical abortions performed during the first trimester of gestation. Adult tissues were obtained from various operations. 5- μ m cryostat sections were prepared from tissue blocks snap frozen in isopentane cooled with liquid nitrogen. Sections were fixed in -20°C acetone for 20 min, washed in phosphate-buffered saline, and employed in the immunofluorescence assay (17).

Cells in Tissue Culture. Fibroblasts [human embryonal fibroblasts (HEF)] were prepared from human fetuses by treating small pieces of body wall tissue with 0.25% trypsin and incubating for 2 h (17). The cells were then washed in culture medium and plated. Cells were maintained in secondary culture in medium RPMI 1640 supplemented with 10% fetal calf serum. 1×10^6 cells were grown on culture dishes (20 cm²) containing small cover slips. The cover slips were harvested after 1-2 days of culture, fixed for 20 min in -20°C acetone, and used in the immunofluorescence assay. The cells were identified as fibroblasts on morphological criteria and by a positive immunofluorescence staining reaction with anti-human collagen and anti-human fibroblast surface-specific antiserum (17). These fibroblast-specific antisera reacted with virtually all cells in the cultures. HeLa cells and Raji leukemia cells were grown and harvested under the same conditions. Normal human lymphocytes were isolated by Isopaque-Ficoll sedimentation (18).

Immunofluorescence Method. Indirect immunofluorescence (IFL) was performed as described previously (17, 19). Purified sheep anti-rabbit IgG antibodies labeled with fluorescein isothiocyanate (fluorescein to protein molar ratio 2.3) were used at a protein concentration of about 10 μg of antibody protein/ml. Standard incubation and washing times were employed. Controls for specificity of staining reactions included normal rabbit sera, rabbit anti-fibroblast surface antiserum (17), anti-collagen antiserum prepared by immunization of a rabbit with collagen eluted from human fetal skin (20), as well as human anti-reticulin antibodies (21, 22). These antisera did not show any reaction with SAA or AA in double diffusion.

Specificity controls also included absorptions of anti-SAA antiserum and purified anti-SAA antibodies with purified amyloid proteins, SAA, and human sera with or without SAA activity detectable by double diffusion. Absorptions were performed by adding equal amounts of antiserum to serially diluted absorbing antigen and incubating at room temperature for 2 h. The absorbed antisera were used in a final concentration four times that of the end-point titer.

Results

SAA-Like Material in Cultured Embryonal Fibroblasts and Sections from Various Connective Tissues. Anti-SAA gave a strong staining reaction with cultured human embryonal fibroblasts. Fibroblasts from four fetuses were tested for up to 30 passages, and in all cases acetone-fixed cells from these cultures exhibited a uniform perinuclear fluorescence (Fig. 1a) with a fine fibrillar appearance at higher magnification (Fig. 1b); virtually all cells in the culture exhibited this staining reaction. In contrast, unfixed cells and paraformaldehyde-fixed cells did not stain. Adult fibroblasts were also used as a substrate for anti-SAA, but this yielded a weaker reaction and, therefore, the

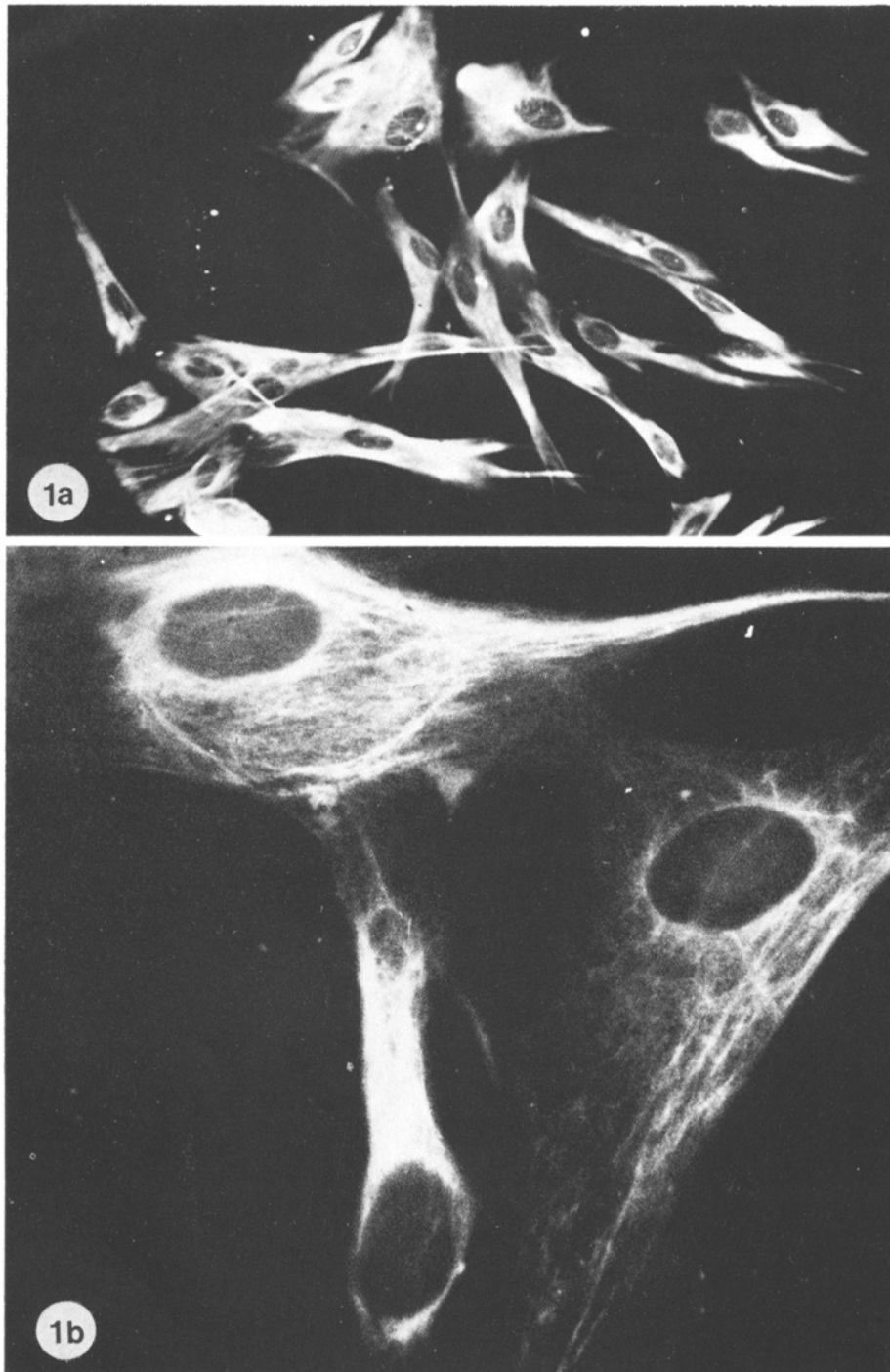


FIG. 1. (a) Embryonal fibroblasts in tissue culture demonstrating fluorescence after indirect immunofluorescent staining with anti-SAA. Acetone-fixed cells, $\times 1,500$. (b) Same fibroblast culture in higher magnification ($\times 4,500$). Note fine fibrillar cytoplasmic staining reaction.

embryonal fibroblasts were selected for the further study involving fibroblast cultures. In similar experiments, no immunofluorescence was observed with the following cell types: human erythrocytes, HeLa cells, peripheral blood lymphocytes, or Raji lymphoid cells. Since the staining reaction appeared to be primarily a property of fibroblasts, sections from various connective tissues were examined. These studies showed that anti-SAA antibodies also reacted with connective tissue fibrils in embryonal skin (Figs. 2 and 3*a*), and in many other organs as will be discussed below. Adult connective tissues were used as substrate for anti-SAA in IFL staining. However, because of marked autofluorescence of elastic or collagen fibers and some nonspecific adherence of serum proteins, it was difficult to interpret the results. Therefore, embryonal skin sections were selected for the specificity investigations together with embryonal fibroblasts.

Specificity of the Anti-SAA Reaction with Skin Sections and Cultured Fibroblasts. The specificity of the reaction with anti-SAA was further investigated with a series of neutralization experiments. Neutralization of anti-SAA immunofluorescent staining was seen after absorption with various preparations of amyloid material (Figs. 3*a-c*) including DAM, purified protein AA as well as purified protein SAA, and all human sera tested which were positive for SAA in double-diffusion tests (Table I). In contrast, no inhibition was seen with the fractions of degraded amyloid which did not contain protein AA, that is, the material excluded from the Sephadex G-100 column (void volume material) (4), or with most human sera negative for SAA. The finding of three sera that inhibited the immunofluorescence but did not have SAA detectable in double diffusion probably reflects a greater sensitivity of the immunofluorescence technique. Serum was obtained from one individual (R. A.) before and after TAB vaccination. The prevaccination serum was negative for SAA in double diffusion and failed to inhibit the staining reaction. Serum obtained 24 h after TAB vaccination was strongly positive for SAA in double diffusion and inhibited, up to a dilution of 160, the immunofluorescence obtained with anti-SAA. Experiments were also performed that showed that the anti-SAA activity could be neutralized by absorptions with 50% suspension of fibroblasts.

Studies with Purified Anti-SAA. Purified anti-SAA antibodies prepared by immunoabsorption on SAA-positive serum insolubilized with glutaraldehyde exhibited a staining reaction on fetal skin sections and on cultured fibroblasts which was indistinguishable from the pattern obtained using whole anti-SAA antiserum as described above. The staining reactions with purified anti-SAA were also completely abolished after absorption with purified protein AA and isolated protein SAA in concentrations similar to those required to neutralize the activity of the anti-SAA antiserum.

Distribution of SAA-Like Material in Various Tissues. Reaction with anti-SAA antiserum was seen with connective tissue fibrils in many organs. A random distribution of fine fibrils was seen particularly in loose connective tissue. Thin twisted fibrils were seen in close association with connective tissue cells (Fig. 2). Fibrils giving a positive staining reaction were well developed in subepithelial connective tissue forming a network of fibrils of different diameters. Thin fibrils were just visible, whereas thick fibrils had a diameter of up to approximately 1 μm .

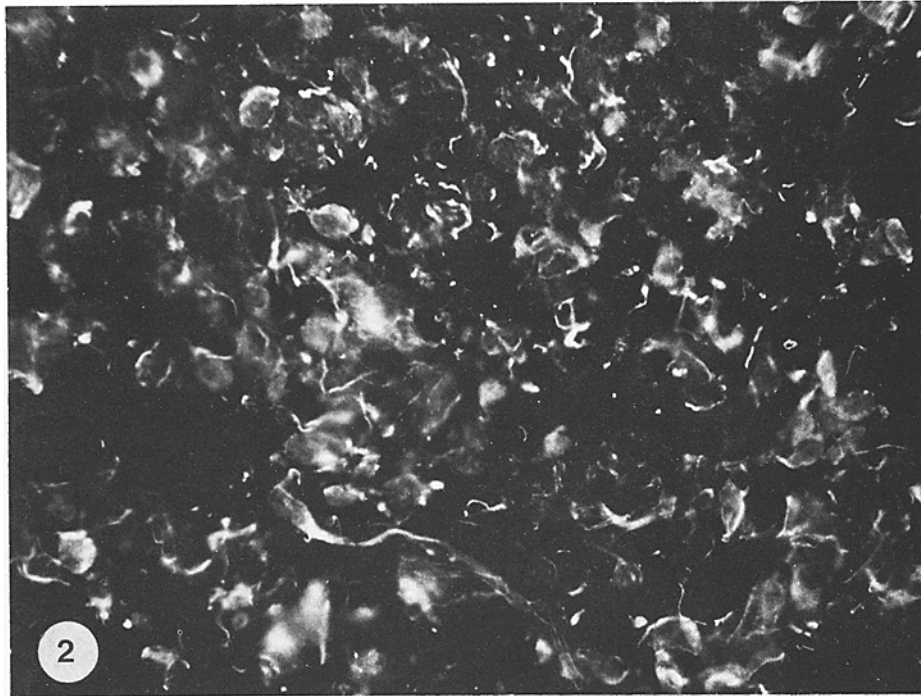


FIG. 2. Fibroblasts in loose connective tissue located in deep dermal layer exhibit short fibrillar fluorescent extensions. IFL staining of acetone-fixed cryostat sections with rabbit anti-SAA. $\times 1,500$.

Fibrils were observed throughout the dermis, both in the papillary and reticular parts. They had the same general orientation and occasionally formed parallel bundles and branching fibrils (Fig. 3*a*). By using phase-contrast illumination, fibrils reacting with anti-SAA could be distinguished (Fig. 3*b*). The pattern of fluorescence seen with the anti-SAA antiserum was different from that obtained with rabbit anti-human collagen and human anti-reticulin antisera. Anti-collagen antiserum stained collagen fibers diffusely throughout the dermal connective tissue (Fig. 3*d*) and no fine fibrils such as those seen with the anti-SAA were observed (Fig. 3*a*).

FIG. 3. (a) Short fluorescent fibrils in the papillary dermis are contrasted to the long fibrils, up to 15 μm with the same general orientation in deeper layers. Epidermis (E) does not stain. Arrow points to fibril seen in Fig. 3*b* with phase contrast. IFL as in Fig. 1. $\times 500$. (b) Occasionally, fibrils corresponding to those stained by IFL can be seen in phase-contrast illumination. Arrow points at fibril stained by anti-SAA in Fig. 3*a*. $\times 500$. (c) Inhibition of fibrillar anti-SAA staining reaction after absorption with SAA-positive serum. There is no fibrillar staining but lumpy precipitates can be seen (arrows) consistent with the antigen-antibody complexes formed because of the absorption. (d) Skin section stained with an anti-collagen antiserum against human fetal skin collagen. Note massive staining in all layers of the dermis, but absence of fluorescence in the epidermis. Both fine coiled fibrils and larger collagen bundles are seen. IFL staining as described in legend of Fig. 1. $\times 500$.

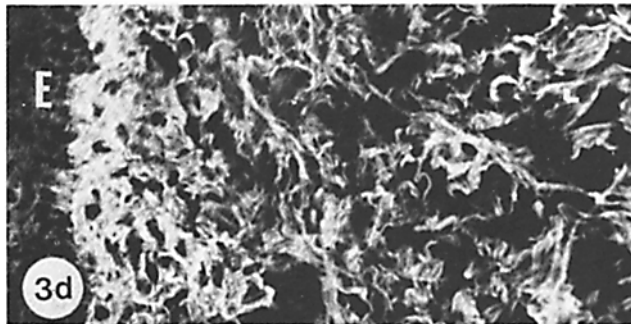
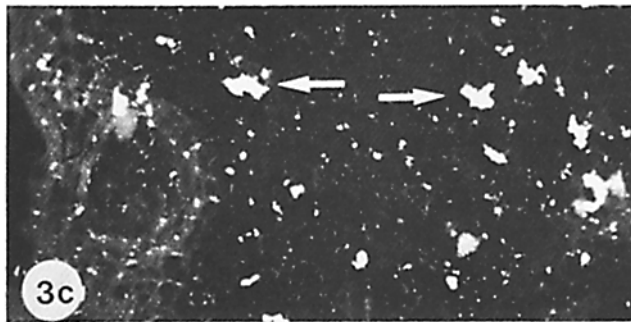
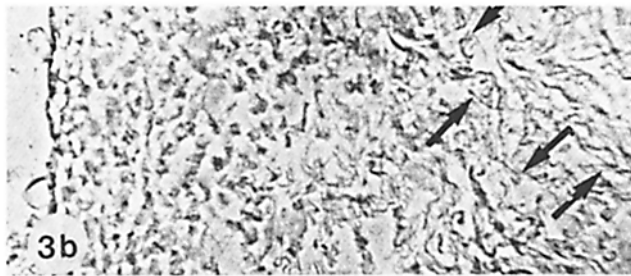
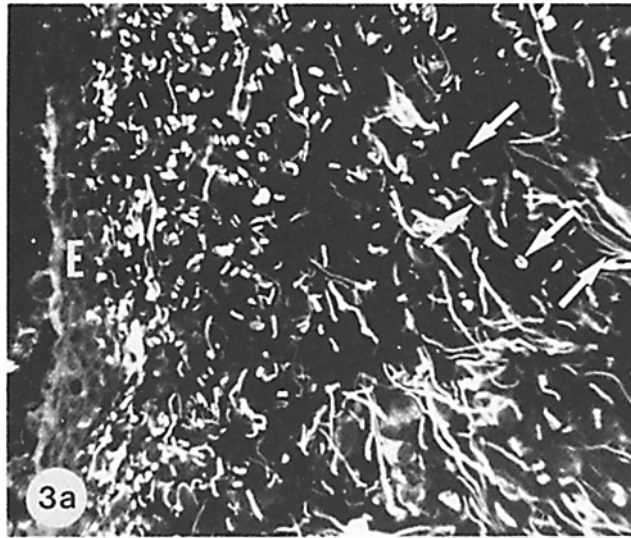


TABLE I
Inhibition of Anti-SAA Antibody Activity with Amyloid Material and Amyloid-Related Serum Component, SAA

Inhibitor	Substrate for immunofluorescence reactions	
	Skin sections	Cultured fibroblasts
Amyloid fibril proteins		
DAM	10-30 μg^*	10-30 μg
DAM fraction I (void volume material)	—	—
Isolated protein AA	1-3 μg	3 μg
Amyloid-related protein SAA		
Isolated protein SAA	2-6 μg	6-8 μg
Serum with high SAA levels	160-320	160
Serum with undetectable SAA levels	—	—

* Indicates lowest concentration of micrograms per milliliter or highest serum dilution giving inhibition; (—), indicates no inhibition at 500 $\mu\text{g}/\text{ml}$ or serum diluted 1/2.

Anti-SAA produced a fine fibrillar fluorescence throughout the wall of the aorta (Fig. 4*a*), and also stained fibrils in the walls of vessels of medium size and big arteries (Fig. 4*b*). In contrast, capillaries and veins did not stain. In the lung strong specific fluorescence was seen in bronchial and bronchiolar walls in close relation to the basement membrane (Fig. 5*a*). This staining was much stronger than the weak reaction seen in the connective tissue between epithelial elements. In the liver, there was a faint staining of sinusoidal walls (Fig. 5*b*), but no staining with hepatocytes or hematopoietic cells. In the kidney, some staining was seen in the interstitium and developing glomerular tufts (Fig. 5*c*). The distribution of staining in some tissues showed similarities with the distribution of elastic fibers.

Discussion

The results indicate that a molecule antigenically related to protein SAA is produced by fibroblasts as identified by morphological criteria, as well as by the reaction with anti-collagen and anti-human fibroblast surface-specific antiserum (17). This protein SAA-related material is normally located as fine fibrils in connective tissues throughout the developing organism. This is consistent with the previous observation that anti-amyloid antibodies produced against DAM reacted with fibroblasts in vitro (23). However, blocking experiments indicated that the staining reactions observed with anti-DAM were not due to only anti-AA antibodies (unpublished results) in spite of the monospecificity of anti-DAM for protein AA in the double-diffusion assay (4, 5). This suggests that DAM preparations contain connective tissue components, unrelated to protein AA, which may not necessarily be integral components of the amyloid fibril. These difficulties could be avoided in the present study in which antiserum to the serum protein of SAA was used. The specificity of the staining obtained with this reagent was demonstrated by the neutralization obtained with isolated protein SAA, SAA-containing sera, DAM, and isolated protein AA.

Fibrils stained by anti-SAA were most abundant in the dermis. The fibers up to 15 μm in length, seemed to have the same general orientation. This suggests that the SAA-like material is a part of the fibrillar connective tissue network.

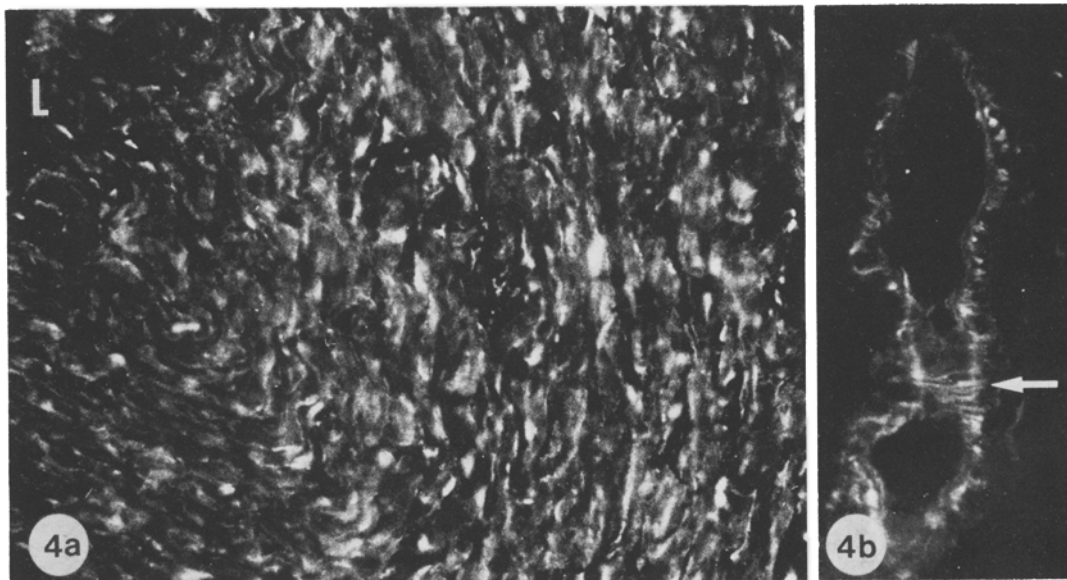


FIG. 4. (a) Cross-section of aorta wall with lumen (L) to the left (L) shows fluorescent fibrils in concentric layers of the muscular coat. IFL with anti-SAA. $\times 1,500$. (b) Medium sized artery in developing kidney partly seen in tangential section (arrow). Fluorescent fibrils seem to form concentric rings in the vessel wall. The fibrils are cut longitudinally at the tangential section (arrow), obliquely at other sites. IFL with anti-SAA. $\times 1,500$.

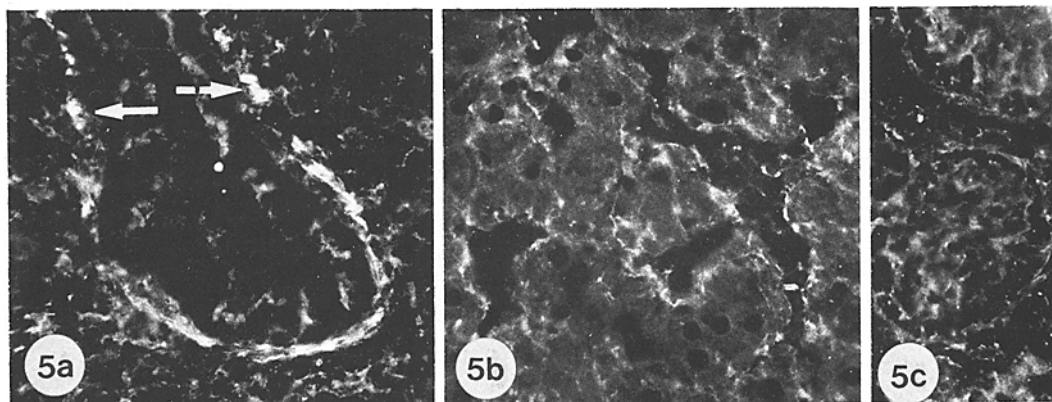


FIG. 5. (a) Bronchiolar wall with bundles of fluorescent fibers with circular arrangement. Cross-section of fluorescent bundle at arrow. IFL with anti-SAA. $\times 1,500$. (b) Faint fibrillar staining reaction in walls of liver sinusoids. IFL as described in legend of Fig. 1. $\times 1,500$. (c) Faint staining reaction in glomerular tuft. IFL as described in legend of Fig. 1. $\times 1,500$.

The distribution of the antigen was clearly distinct from the patterns produced by anti-collagen antibodies (20), anti-reticulin antibodies (21, 22), and antibodies to the connective tissue SF antigen of fibroblast origin (24). Localization of the SAA-like material, for example in vessel walls, corresponded rather well to the histochemical staining for elastin (25). However, in the fetal skin only faint histochemical staining corresponded to the distribution of fluorescence.

The known amino acid composition and sequence data for SAA and AA (1-3, 9, 10) proteins show no relationship to elastin (26-28). However, a relationship to the microfibrillar component of the elastic fiber cannot be ruled out on the available data (27, 28). A possible relationship of amyloid fibril proteins to connective tissue microfibrils has been suggested previously (29). Recently, a structural glycoprotein isolated from skin has been shown to form amyloid-like fibrils *in vitro* (30). This protein had an amino acid composition which was similar to that of protein SAA and was found in higher amounts in newborn than in adult skin (30).

The fibrils reacting with anti-SAA antibodies varied slightly in appearance in different tissues. In loose connective tissues it was occasionally possible to observe fibrillar extensions of connective tissue cells. This is consistent with the staining of fibroblasts *in vitro*, even if extracellular fibrillar extensions could not be seen. Apparently the staining reaction seen with fibroblasts *in vitro* represents intracellular SAA-like material. Lack of fibril formation *in vitro* does not necessarily indicate that the secreted molecule does not form fibrils *in vivo*. This has been shown convincingly in the case of collagen synthesis where soluble procollagen molecules start to polymerize only after extracellular enzymatic cleavage of terminal extension peptides (31). In this respect collagen and possibly the SAA-like material differ from the fibroblast SF antigen which forms long fibrillar extensions under normal tissue culture conditions (32). Fibril formation may be a complex process influenced not only by enzymes in the medium but also by the presence of other macromolecules such as glycosaminoglycans (33) in developing connective tissues.

The structural relationship of the connective tissue component observed in these studies to protein SAA and protein AA remains to be defined. The finding that inhibitors of protein synthesis do not modify the SAA response to endotoxin³ indicates that the increase in the concentration of SAA during acute inflammatory episodes is not due to synthesis of SAA *de novo*. An alternative explanation in line with the observations reported here, is that SAA is released into the circulation by proteolysis from a larger precursor molecule. This may be the connective tissue component detected by the immunofluorescent studies documented here.

Summary

Protein SAA is a serum protein related to the major constituent of secondary amyloid fibrils, protein AA, and has been suggested to be a precursor of the amyloid protein AA. In the present study, the origin of SAA was investigated by studying human fetal tissues and cultured human fetal fibroblasts with the indirect immunofluorescence technique. Anti-SAA antibodies reacted strongly with cultured fibroblasts producing a fine fibrillar cytoplasmic staining and with extracellular fibrils in loose connective tissues and vessel walls. The reactions were specifically inhibited by absorption with degraded amyloid material, isolated protein AA, isolated protein SAA, and sera from patients with elevated levels of SAA. In contrast, no inhibition was seen with amyloid fibril

³ Anders, R. F., and J. B. Natvig, Manuscript in preparation.

material devoid of AA protein or by human sera in which SAA was not detectable by double-diffusion tests. These observations showed that SAA-like material is produced by fibroblasts and indicate that it is a normal constituent of developing extracellular connective tissue fibers.

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